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Molecular cloning and characterization of *twist* gene in *Bombyx mori*

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Abstract The *twist* genes are an evolutionarily conserved group of regulatory basic helix–loop–helix (bHLH) transcription factors. In present study, the *twist* gene was firstly cloned from *Bombyx mori* and was designated as BmTwist. Sequence analysis showed that BmTwist cDNA contains a 798 bp open reading frame, encoding a peptide of 266 amino acid residues. Sequence alignment showed that BmTwist protein shared extensive homology with other invertebrate Twist proteins in bHLH motif. RT-PCR and western blot analyses revealed that BmTwist expressed in all developmental stages of *B. mori* larvae various larval tissues. Here the authors also presented the results of prokaryotic expression, purification, and polyclonal antibody production of the BmTwist protein. Immunofluorescence of BmTwist in BmN cells using the antibodies showed that BmTwist protein was located in both the nucleus and cytoplasm. Furthermore, using *B. mori* nuclear polyhedrosis virus (BmNPV) baculovirus expression system, the authors expressed a recombinant *twist* protein in BmN cell line. The obtained results, especially the preparation of polyclonal antibodies against BmTwist, will greatly facilitate further studies to explore biological functions of BmTwist protein such as identifying its potential binding partners.

Keywords Twist · *B. mori* · Bioinformatics · Sub-cellular localization · PCR

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Introduction

The basic helix–loop–helix transcription factors are present in animals, plants, and fungi, and play important roles in a variety of regulatory processes in organisms. The bHLH motif was first identified by Murre et al. in two murine transcription factors E12 and E47. The bHLH domain is approximately 60 amino acids in length and comprises a DNA-binding basic region of 15 amino acids followed by two α-helices separated by a variable loop region [1, 2].

Several bHLH genes have been identified in various organisms including both vertebrates and invertebrates. Recently, in silkworm (*B. mori*), 52 bHLH genes have been identified [3]. Phylogenetic analysis has classified these 52 bHLH genes into 39 bHLH families and six groups.

The *twist* gene, which belongs to the Twist family of bHLH transcription factors, was first identified in *Drosophila* as a mutant embryo with a twisted torso [4]. Subsequently, several orthologs of *Drosophila* Twist have been identified in vertebrates: *Xenopus* (*X-Twist,* [5]), mouse (*M-Twist*, [6]), and human (*H-Twist*, [7]), as well as a Twist-related factor in *Caenorhabditis elegans* (*Ce-Twist*, [8]). However, no reports have been published on *twist* gene in *B. mori* till now.

The Twist protein is a highly conserved transcription factor, which recognizes and binds to the E-box of the promoter of responsive genes. Expression of Twist has been implicated in the inhibition of differentiation of multiple cell lineages including muscle, cartilage, and bone cells [9]. Moreover, in the period of gastrulation, Twist is necessary for the allocation of mesodermal cells to particular tissue fates, including somatic mesoderm, heart, visceral mesoderm, fat body, and mesodermal glia. During this period, Twist activates another set of genes that are necessary for myogenesis [10, 11]. It has also been

reported that Twist is closely related to invasion, metastasis, and proliferation of many cancer cells [12, 13]. Therefore, investigations of Twist family members may bring new insights into cancer research.

The domesticated silkworm, *B. mori*, is an important lepidopteran model insect. The silkworm genome-sequencing work had been completed in October 2003, and the identification of its bHLH family members and gene electronic cloning had been completed in April 2008 in our laboratory. However, the bHLH transcription factor genes in *B. mori* on experimental aspects have not yet been reported.

In this study, *B. mori twist* (BmTwist) gene was cloned and expressed in *E. coli*. The expressed Twist protein was purified and used to prepare polyclonal antibody. The authors also identified the overexpressed Twist protein in BmN cell line by Western blot. Moreover, the expression profiles of BmTwist gene in various tissues and subcellular localization in BmN cells were investigated. The obtained results would facilitate future studies on elucidating biological functions of Twist protein.

Materials and methods

Materials

B. mori strain C108 (Standard strain of silkworm) and the *B. mori* cell line BmN were maintained in our laboratory. The larvae were reared with fresh mulberry leaves at 25 ± 2°C under a 12 h -light/-dark photoperiod. The *B. mori* cell line BmN derived from ovary of silkworm [14] was cultured at 27°C in TC-100 insect medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, USA).

All primers, RNase-free DNaseI, EX-Taq polymerase, restriction enzymes, T4 DNA ligase, and the DNA subcloning vector pMD18-T, were purchased from TaKaRa (Dalian, China). Chemicals were purchased from Sigma (MO, USA) or a domestic provider in China if not stated otherwise.

Bioinformatics analysis

The full length of BmTwist gene was obtained by searching silkworm EST database with BmTwist bHLH motif. To establish the genomic organization, the cDNA sequence was blasted to the contigs of *B. mori* genome in the GenBank. SIM4 (http://pbil.univ-lyon1.fr/sim4.php) was used to align the cDNA sequence with the genomic sequences to search for potential introns. The ExPASy Translate tool (http://au.expasy.org/tools/dna.html) was used to deduce the cDNA's amino acid sequence. Multiple sequence alignment of proteins was performed with the Clustal W program [15], and edited with Genedoc. A phylogenetic tree based on the multiple sequence alignment was constructed by the neighbor-joining method [16] using the MEGA4.1 program [17]. Another silkworm cDNA database (http://silkworm.genomics.org.cn/) was used to analyze the ORF and obtain the map of gene location on chromosome.

RNA extraction and transcriptional analysis of BmTwist

The larvae at different developmental stages, and various tissues (hemolymph, testis, ovary, epidermis, silk gland and midgut) of the fifth instar larvae of silkworm strain C108 were dissected, immediately frozen in liquid N_2, and stored at −80°C for later use. Total RNA was extracted from frozen samples with RNeasy mini kit (Qiagen), treated for 20 min at 37°C with RNase-free DNaseI (TaKaRa), purified with phenol–chloroform, precipitated with ethanol, and finally dissolved in DEPC-treated ddH_2O. cDNAs were generated from these RNA using M-MLV RTase (Promega) and an oligo-DT primer according to the manufacturer's instructions. PCR was then carried out using BmTwist-specific primers: 5′-CG**GGATCC**ATGAATTA CGATAACTGTGACAATAGA-3′ and 5′-CC**CTCGAG** TCACGATTGTCCGTTGGTC-3′, where the underlined characters were restriction enzyme sites of BamHI and XhoI, respectively. A 284-bp fragment of *B. mori* cytoplasmic actin gene A3 was amplified in parallel with each RNA sample using the following primers: Bm-actinA3-F—5′-GCGCGGCTACTCGTTCACTACC-3′, and Bm-actinA3-R—5′-GGATGTCCACGTCGCACTTCA-3′ as an internal control for adjustment of template RNA quantity. The PCR reaction was carried out for 35 amplification cycles (94°C/60 s, 53°C/45 s, and 72°C/50 s) in a Gene Amp 2400 System thermocycler. 10 µl of each PCR product was electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining.

Protein expression in *E. coli*

The above PCR product was ligated into pMD18-T vector (TaKaRa) using T4 DNA ligase (TaKaRa). Positive clones were isolated and sequenced, then subcloned into the pGEX-4T-2 expression vector and transformed into *E. coli* strain Rosetta cells. The transformants harboring the recombinant plasmid were confirmed by restriction enzyme analysis.

To express the recombinant protein, cells of a fresh transformant were cultured in LB medium containing 100 µg/ml ampicillin and grown overnight at 37°C and 250 rpm. This overnight culture was then transferred to fresh LB medium containing ampicillin of the same

concentration. The cultures were grown at 37°C and 250 rpm until OD600 reach 0.6. Expression of the GST fusion protein was induced with IPTG (final concentration 0.05–0.4 mM during optimization) and further cultured at 16°C for another 10 h. Cells were harvested by centrifugation (4500 g, 4°C, 15 min) and 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the recombinant protein. SDS-PAGE was performed in the Mini-Protein system (Bio-Rad, USA). After electrophoresis, the gel was stained with Coomassie brilliant blue R250 to visualize the protein bands.

Preparation of specific polyclonal antibodies

The GST-tagged recombinant BmTwist protein was purified on a Glutathione Sepharose 4B column (GE Healthcare) and used to raise polyclonal antibodies in New Zealand white rabbits. The antibody was prepared using standard techniques [18]. BmTwist protein (about 2 mg) was injected subcutaneously to immunize New Zealand white rabbits in complete Freud's adjuvant, followed by two booster injections in incomplete Freud's adjuvant. After the last injection, blood samples were taken from the marginal vein of the rabbit ear. After centrifugation, the sera were obtained to determine the antibody titer by ELISA.

Antiserum titer determination by ELISA

The titers of antiserum were examined using an indirect enzyme-linked immunosorbent assay (ELISA). In short, the wells of polystyrene microtiter plates (Greiner Bio-One, USA) were coated with 150 μl antigen (2 μg/ml) in carbonate–bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). After incubation overnight at 4°C, the wells were washed three times with PBS–Tween buffer (0.05% Tween 20 in PBS). The coated wells were blocked with 200 μl of 3% BSA for 1 h at 37°C and then incubated with 150 μl polyclonal antibodies against BmTwist with different deliquations (from 1:5000 to 1:640000) at 37°C for 1 h. After washing with PBST for four times, the plates were incubated with goat anti-rabbit IgG conjugated with peroxidase at 37°C for 1 h. TMB substrate (100 μl per well) was added and incubated for 15 min after washing with PBST. 50 μl of stop buffer (2 M H_2SO4) was added to each well, and the optical density (OD) was read at 450 nm using an ELISA reader [19].

Immunofluorescence microscopy

For immunofluorescence staining, BmN cells, without any exogenous gene expression, grown on sterile coverslips were washed with PBS, and fixed with 2 ml of 4% paraformaldehyde in PBS for 20 min. Then cells were washed three times with PBS, permeabilized with 0.2% Triton X-100 in PBS for 15 min, and further incubated in blocking buffer (1% skimmed milk powder in PBS) for 1 h. After washing four times with cold PBS, cells were incubated with BmTwist polyclonal antiserum (1:1000) for 2 h at 37°C. Cells were washed three times with PBS and then incubated with the secondary antibody, fluorescein isothiocyanate (FITC)- conjugated goat anti-rabbit IgG (1:3000) (Qualex, Inc), for 1 h at 37°C. Cells were then washed three times with PBS. After staining nuclear DNA with DAPI for 15 min, cells were examined with a confocal laser scanning microscope (Zeiss lsm 5 live). In negative controls, the BmN cells reacted with preimmune serum as the primary antibody.

Expression of *twist* gene in BmN cells and western blot analysis

A bac-to-bac/BmNPV baculovirus expression system was used for expression of BmTwist [20]. In brief, the twist cDNA was inserted into pFastBacHTc at the BamHI/XhoI sites and transformed to *E. coli* DH10Bac/BmNPV. Through inside transposition in bacteria, the twist gene was transferred to a baculovirus shuttle vector (bacmid) within a mini-att Tn7 target site and formed a recombinant bacmid baculovirus containing target gene. Then screened by blue/white selection, the positive recombinant bacmid baculoviruses were transfected into BmN cells, and the supernatants were harvested.

Western blot was performed as described previously [21]. After 15% SDS-PAGE, the gel was immersed in cold transfer buffer (0.025 M Tris, 0.19 M Glycine, 20% methanol), and the proteins were transferred to polyvilinidendifluoride (PVDF) membrane (Millipore). The membranes were subsequently blocked in 4% skimmed milk powder in PBST for 3 h, and immunoblotted with the anti-BmTwist polyclonal antiserum diluted 1:1000 for 1.5 h at room temperature. After washing with PBST for five times, the membrane was incubated with peroxidase-conjugated goat anti-rabbit IgG diluted 1:2000 for 1.5 h at room temperature. After washing with PBS for five times, the bands were visualized using diaminobenzidine(DAB)or the enhanced chemoluminescence (ECL) method (Thermo Scientific Pierce).

Results

Bioinformatics analysis of BmTwist

Using tblastn program, the authors searched against silkworm EST fragments with BmTwist bHLH motif, then the authors assembled these EST fragments, obtained 5′-UTR

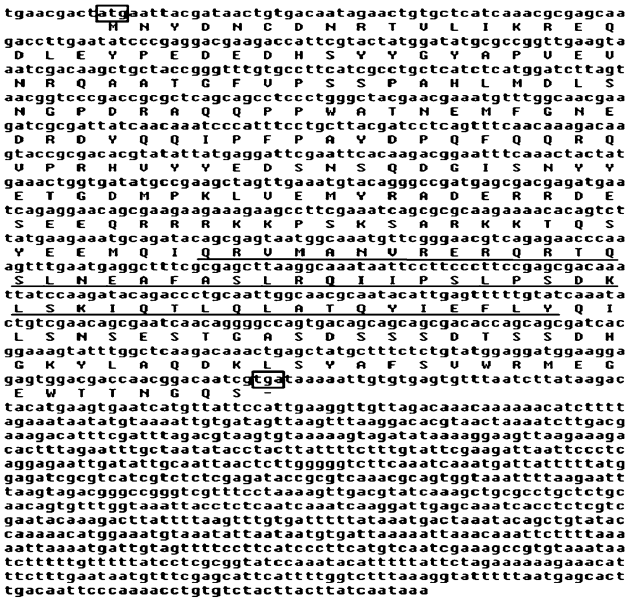

Fig. 1 Nucleotide sequence and deduced amino acid sequence of *Bmtwist* gene. The predicted amino acid sequence is represented by one-letter code underneath the nucleotide sequence. The initiation and stop codons are framed, and the conserved RNA binding domain is underlined

and ORF sequence of BmTwist, and predicted the 3′-UTR with GenScan (http://genes.mit.edu/GENSCAN.html) and GeneBuilder (http://www.bioscience.org/urllists/genefind.htm). The ORF begins with the initiation codon ATG at position 72 bp, ending with TAG at 869 bp, and there is no intron in the coding region (Figs. 1, 2). Multiple sequence alignment of Twist proteins among *B. mori* and other nine species revealed that these proteins were highly conservative in the bHLH motif and its flanking regions as well as in the N terminus (Fig. 3). The phylogenetic tree of BmTwist was constructed with MEGA4 (Fig. 4). Using silkworm cDNA database (http://silkworm.genomics.org.cn/) to search the gene location, BmTwist was found to be located on chromosome 3 of *B. mori* genome (data not shown).

Expression and identification of recombinant protein

BmTwist was subcloned into the pGEX-4T-2 prokaryotic expression vector. Positive clones were isolated and transformed into *Escherichia coli* strain Rosetta cells. The authors induced the expression of BmTwist at 16°C with various amounts of IPTG (Fig. 5a), and both soluble and insoluble BmTwist proteins were produced. The expression of GST-tagged BmTwist was also confirmed by anti-GST monoclonal antibody. Western blot analysis using anti-GST antiserum confirmed that the 54-kDa protein was the fusion protein (Fig. 5b, lane 2), while GST protein was only 26 kDa (Fig. 5b, lane 1). The purified fusion protein was used to immunize rabbits to produce the specific antiserum against BmTwist.

Titer and specificity analysis by ELISA and Western blot

After immunizing rabbits with GST-BmTwist fusion protein according to the standard protocol, antibody titers were determined by ELISA and were found to be approximately 1: 160000 (data not shown). At the same time, pre-immunized rabbit serum used as a negative control did not result in a detectable signal. Western blot analysis with the polyclonal antibodies raised against the BmTwist detected a ~29-kDa protein in BmN cell lysate overexpressed with BmTwist by transfection (Fig. 7, lane 1), which also indicated that the obtained antibody could serve as a good tool to characterize BmTwist protein.

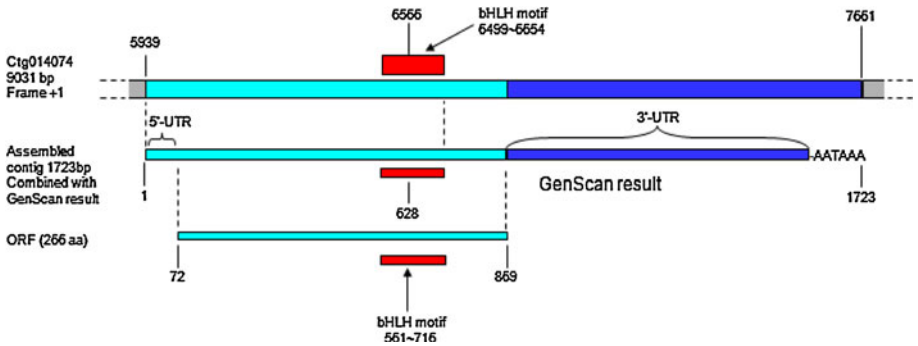

Fig. 2 Genomic-coding region and open-reading frame (ORF) of *B. mori twist* gene. The predicted cDNA length is 1723 bp, with its coding region from nucleotides nos. 5939–7661 on ctg014074 in *B. mori* genomic database. The cDNA includes a 5′-UTR of 71 nucleotides and a 3′-UTR of 854 nucleotides. Its ORF consists of 798 bp and codes for a protein of 266 amino acids residues. The bHLH motif is coded by nucleotides nos. 561–716 on the cDNA

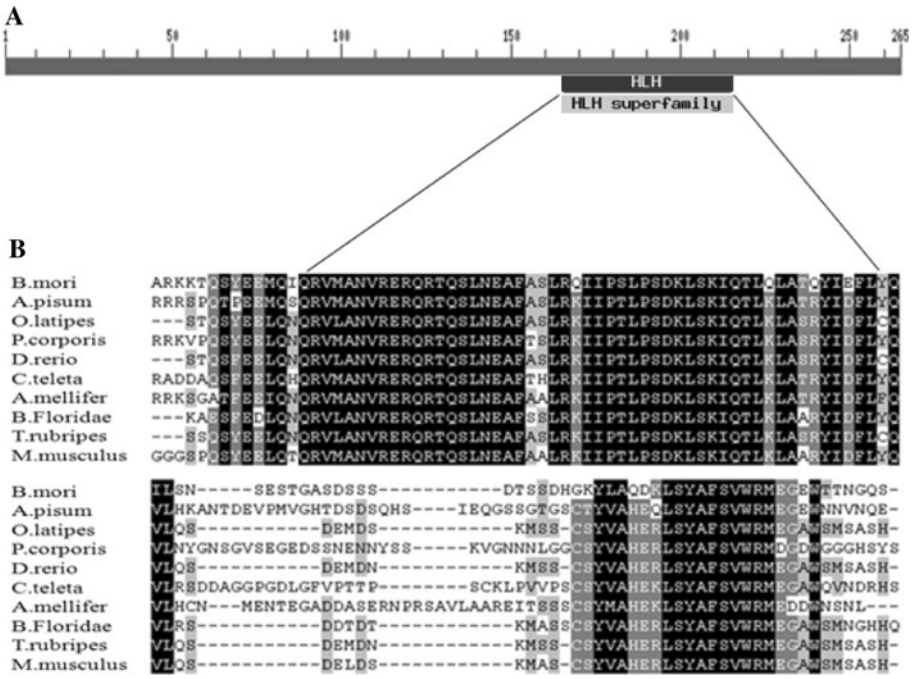

Fig. 3 Sequence alignment of the conserved domain of Twist proteins. **a** Conserved domain of Twist protein in *B. mori*. bHLH motif is located within amino acid residues 164–215. **b** Multiple sequence alignment of the most conserved regions of Twist proteins among *B. mori* and nine other species. The sequences were shaded using GeneDoc. Conserved amino acids exist in the bHLH motif and the latter's flanking regions as well as in the N terminus. The sources of sequences and their GenBank accession numbers are *Acyrthosiphon pisum* (NP_001155424), *Oryzias latipes* (DAA06067), *Pediculus humanus corporis* (XP_002428670), *Danio rerio* (NP_001005956), *Capitella teleta* (ABP35759), *Apis mellifera* (NP_001011637), *Branchiostoma floridae* (XP_002606170), *Takifugu rubripes* (NP_001098070), and *Mus musculus* (EDL36871)

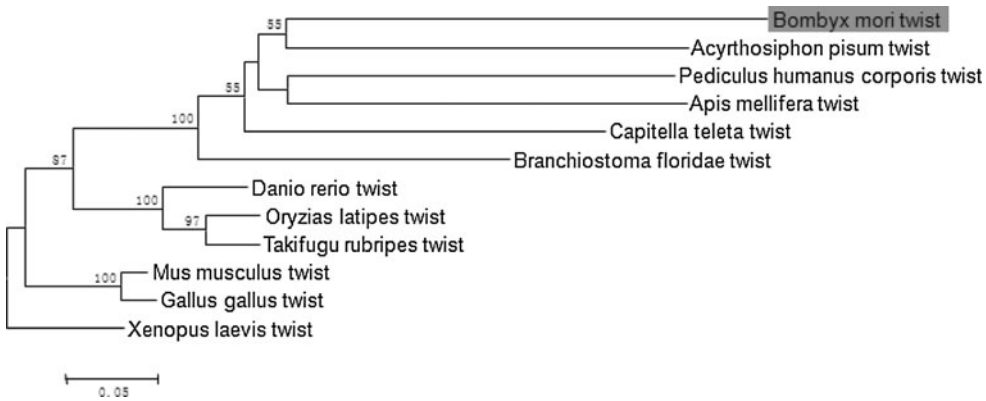

Fig. 4 Phylogenetic relationship of Twist proteins between *B. mori* and 11 other species. A neighbor-joining (NJ) tree is shown. For simplicity, bootstrap values less than 50 are not shown. The Twist sequence of *Xenopus* was used as the outgroup. The scale *bar* indicates percentage of amino acid substitution as per unit branch length. The sequences used in phylogenetic tree analysis were *Acyrthosiphon pisum* (NP_001155424), *Oryzias latipes* (DAA06067), *Pediculus humanus corporis* (XP_002428670), *Danio rerio* (NP_001005956), *Capitella teleta* (ABP35759), *Apis mellifera* (NP_001011637), *Branchiostoma floridae* (XP_002606170), *Takifugu rubripes* (NP_001098070), *Mus musculus* (EDL36871), *Gallus gallus* (NP_990070), and *Xenopus* (NP_001091211)

Expressional analysis of BmTwist at various developmental stages and in different tissues

The levels of BmTwist transcript at various developmental stages of silkworm were examined by RT-PCR. The results indicated that mRNA was detectable from egg to the fifth instar larvae (Fig. 6a). To investigate the expression profile of BmTwist, the total RNA from the fifth instar larvae was isolated from hemocytes, testis, ovary, epidermis, silk gland, and midgut, and subjected to RT-PCR. The results

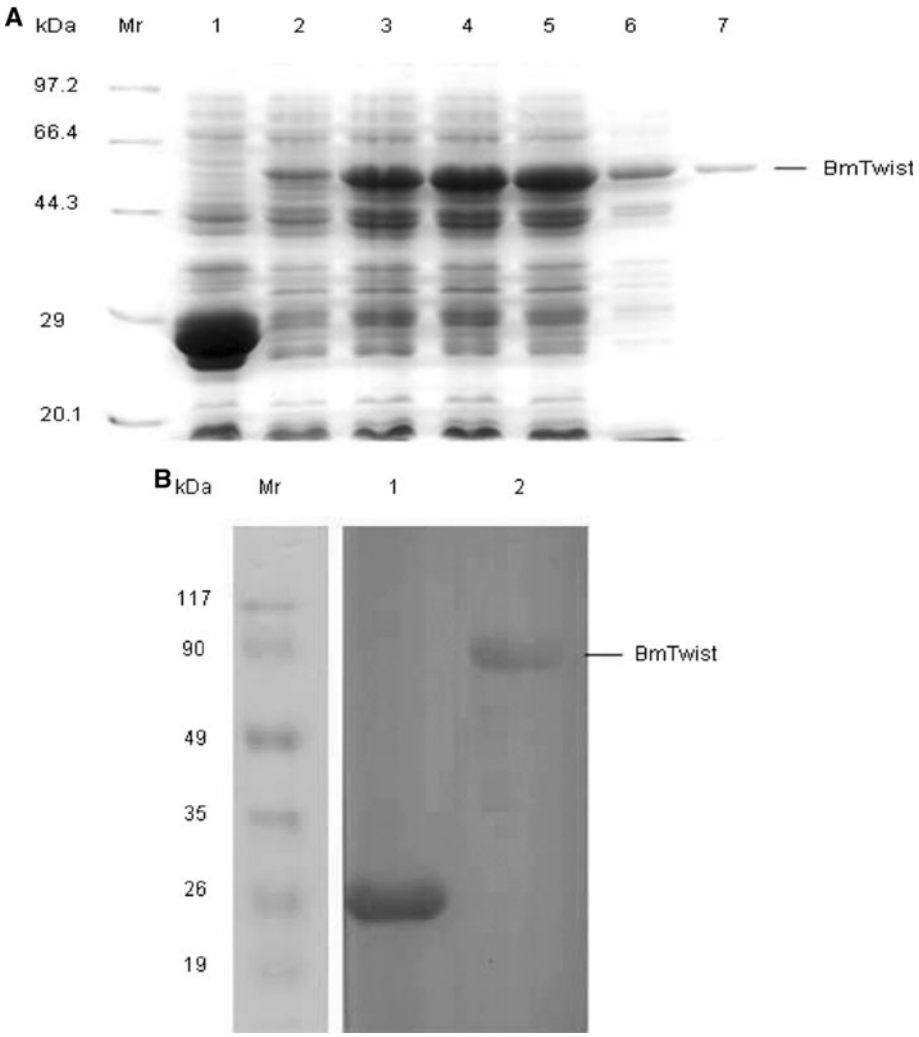

Fig. 5 SDS-PAGE stained with Commassie blue and Western blot analysis of recombinant BmTwist. **a** *Lane 1* lysate of host cells transformed with empty vector; *lanes 2–5* lysate of rosetta cells harboring pGEX-4T-2-BmTwist induced with 0.05, 0.1, 0.2, and 0.4 mM IPTG; *lane 6* soluble fractions of total cell lysate; *lane 7* recombinant BmTwist purified by GST 4B column. **b** Western blot analysis of recombinant BmTwist with GST antibody. *Lane 1* protein extracts of transformed cells with pGEX-4T-2; *lane 2* protein extracts of transformed cells with GST-BmTwist. The bands were visualized through DAB method

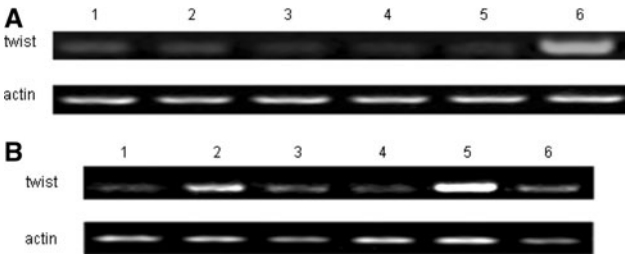

Fig. 6 **a** RT-PCR analysis of silkworm *twist* gene at various developmental stages. *Lane 1* egg; *lanes 2–6* larvae of instar 1, 2, 3, 4, and 5, respectively. **b** Expression of twist mRNA in various *B. mori* tissues. *Lanes 1–6* hemolymph, testis, ovary, epidermis, silk gland, and midgut, respectively. Total RNA from each tissue was used for cDNA synthesis. PCR products amplified with gene-specific primers for twist and *Bm-actin A3* were analyzed on 1% agarose gels and visualized by ethidium bromide staining

showed that BmTwist gene expression could be detected in all the tissues (Fig. 6b). Western blot analysis of different tissues further confirmed the presence of BmTwist in hemolymph, silk gland, fat body, ovary, midgut, and trachea (Fig. 7, lanes 2–7).

Subcellular localization of BmTwist in BmN cells by CLSM

The intracellular localization of BmTwist was determined by immunofluorescence using prepared anti-BmTwist serum. BmTwist was seen in both cytoplasm and nucleus, predominately in the cytoplasm in perinuclear area (Fig. 8).

Discussion

A superfamily of transcription factors containing basic helix–loop–helix (bHLH) domain plays important roles in the control of cellular proliferation, tissue differentiation, and development in animals, plants, and fungi [22]. It has

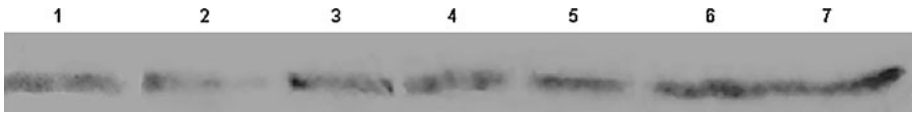

Fig. 7 Western blot analysis of BmTwist protein in BmN cells and different *B. mori* tissues. *Lane 1* the overexpressed Twist protein in BmN cell line, *Lanes 2–7* hemolymph, silk gland, fat body, ovary, midgut, and trachea, respectively. The bands were visualized by ECL (Thermo Scientific Pierce) method

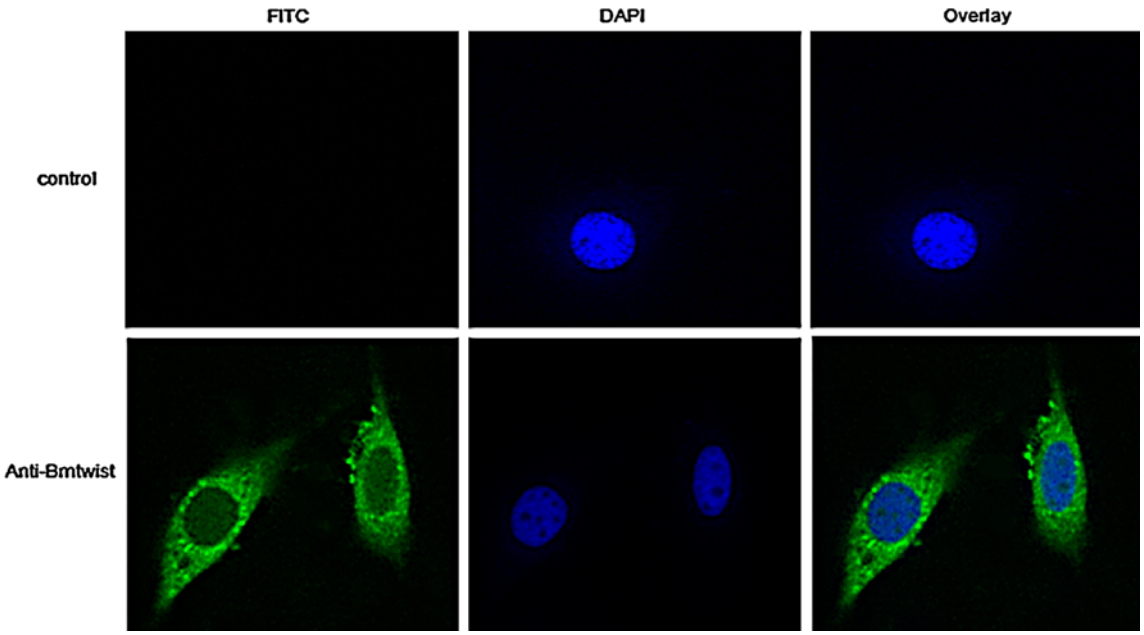

Fig. 8 Subcellular localization of BmTwist in BmN cells. BmN cells were treated with anti-BmTwist antibody, followed by treatment with FITC-conjugated goat anti-rabbit IgG, and the nucleus was treated with DAPI (*blue*), which were examined under confocal laser scanning microscope. From *left to right* green fluorescence for FITC-treated BmTwist, DAPI-treated nucleus and the overlay images. For a control, pre-immune serum was used as the primary antibody

been reported that Twist may serve as a useful molecular marker for carcinoma and an indicator for tumor metastasis [12, 13]. Twist might also be a useful therapeutic target to prevent carcinoma or inhibit its malignant progression as well as metastasis. Through nucleotide sequence analysis, the authors found that *B. mori* Twist gene contained a 798-bp ORF encoding a peptide of 266 amino acid residues, in which there was a bHLH motif, and this was consistent with DNA sequencing; this further indicated that there are structurally conserved bHLH domains in the coding region. Moreover, sequence alignment suggested that the BmTwist had high homology with the other species twist proteins in bHLH domain, indicating a highly conserved and similar role for these twist.

This is the first report about cloning and characterization of a silkworm Twist gene. The successful expression of BmTwist protein in *E. coli* and the preparation of the polyclonal antibody will provide a substantial base for further clarification of the BmTwist function. Furthermore, the authors identified the overexpressed Twist protein in BmN cell line by western blot. The authors also performed RT-PCR and confirmed extensive expression of this gene in different silkworm tissues and at different developmental stages. Moreover, the authors have come to notice that the protein was located in both cytoplasm and nucleus, predominately in the cytoplasm in perinuclear area. For being a bHLH transcription factor, Twist protein appears to be regulated primarily at the level of its subcellular localization [23, 24], other than transcriptional regulation [25]. Specific sequences have been reported to be responsible for nucleocytoplasmic transport. The classical nuclear export sequence (NES) is leucine-rich [26] and Isoleucine-rich [27, 28] region. From BmTwist protein sequence, the authors also found that there are many isoleucines and leucines in the N terminus. Further, integrin-mediated adhesion has been implicated to be involved in the Twist nuclear translocation [29]. Thus, loss of integrin-mediated adhesion may be a cause to the cytoplasm accumulation of Twist in BmN cells, which remains to be further elucidated.

In conclusion, some of the bHLH proteins such as Twist show well-conserved function between insects and humans and these proteins are known to play key roles in causing human diseases such as cancer. *B. mori* could provide a simpler model system to enable us study the function of these proteins.

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